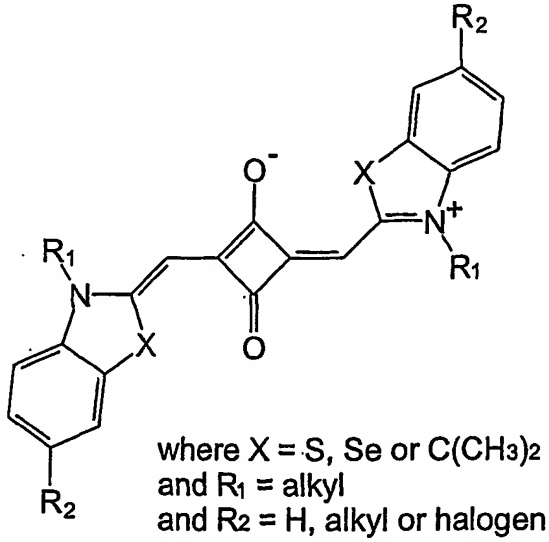
Fluorescent Compound

The present invention relates to novel photoluminescent compounds, to a novel protein detector and to a novel method of detecting the presence of protein in a fluid, in particular water, or bodily fluids.

Photoluminescent compounds are known, and known photoluminescent compounds include the class of dyes known as squaraine dyes, in particular the indolenine series of squaraine dyes, which are substituted and unsubstituted compounds of the general formula:



where X = S, Se or $C(CH_3)_2$
and R_1 = alkyl
and R_2 = H, alkyl or halogen

Known indolenine squaraine dyes include both symmetrical and asymmetrical compounds, and are described, for example, in an article by E. Terpetschnig et al. in Anal. Chim. Acta 282 (1993) pages 633 - 641.

These dyes are insoluble in water and are used, inter alia, for the qualitative detection of the presence of protein in fluids. In order to use these dyes for the detection of the presence of proteins, which are themselves water-soluble in a fluid, in particular an aqueous fluid, the dye has to be dissolved in a solvent comprising a mixture of water and an alcohol such as methanol. When protein is added to a water/methanol solution of an indolenine squaraine dye, there is an increase in fluorescence. The analytical method disclosed in the article cited above is a purely qualitative method, simply recording the presence or absence of protein in the test fluid sample, and cannot therefore be used to determine the amount of protein present.

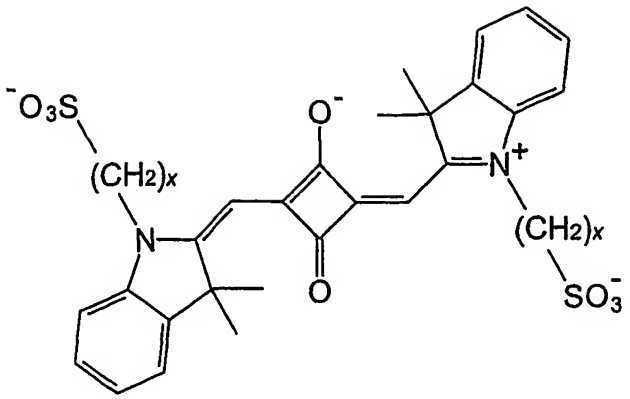
Current methods for the quantitative analysis of protein in fluids and semiquantitatively / quantitatively as stains for proteins in gels include the Bradford [M.M. Bradford in Anal. Biochem. 72 (1976) 248], Lowry [O.H. Lowry et al. in J. Biol. Chem. 193 (1951) 265] methods and bicinchoninic acid (BCA) [P.K. Smith et al. in Anal. Biochem. 150 (1985) 76] methods for fluids and silver stain [C.R. Merril in Meth. Enzymol. 182 (1990) 477] and Coomassie Blue [S. Fazekas de St. Groth et al. in Biochim. Biophys. Acta 71 (1963) 377] for gels. The Bradford, Lowry and BCA methods are colourimetric techinques (ie. colour change) and are suitable for solution protein ranges of 8 – 2000 μg/mL, require multiple reagents and incubation times and suffer from interference from numerous common reagents. Coomassie Blue, used in the Bradford method, takes 20 – 30 mins and is not hindered by tris buffers. Silver stain is a very sensitive (10 – 100 ng/mL) chromatogenic-technique and is used as a gel stain but care must be taken in the handling of the gel and the whole technique

takes 2 – 3 hours. Coomassie Blue is also a gel stain and can be used instead of silver stain if the protein range is in the chromatogenic μg range. Other commercial techniques for the detection of protein concentration include the NanoOrange™ Protein Quantitation Kit and the CBQCA™ Protein Quantitation Kit, both from Molecular Probes, which are ultra-sensitive solution techniques reliant on changes in fluorescence but are not linear over the ranges given for the squaraine derivative. These two techniques also require 30 mins preparation time.

It is an object of the present invention to provide a novel photoluminescent compound, in particular a novel photoluminescent compound that is soluble in water, or water containing solvent mixtures, and retains its photoluminescent properties in these media.

It is a further object of the present invention to provide a method for detecting the presence of protein in fluids, or as a gel stain, in which the above time constraints are reduced or substantially obviated and at a greater sensitivity.

The present invention provides water-soluble photoluminescent compounds including a symmetrical skeletal structure of the formula



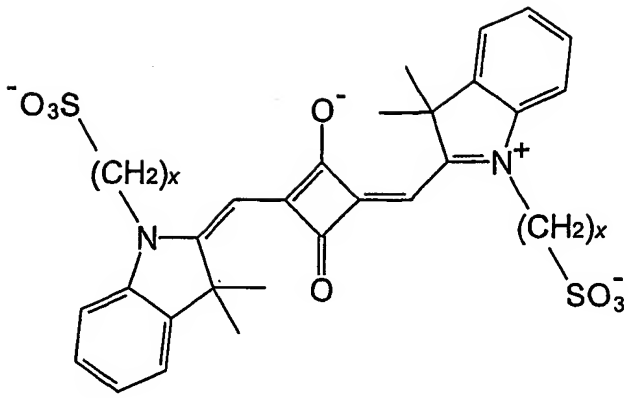
in which x may represent any integer, and in which the phenyl rings may be substituted.

Particularly preferred compounds according to the invention include those wherein $x = 3$.

Further preferred compounds according to the invention include those which are unsubstituted or in which one or both phenyl rings are substituted in the 5-position with alkyl (including ^{i}propyl and ^{t}butyl) or halogen groups.

Particularly preferred compounds according to the invention include 2,4-bis(1-(propan-3-sulfonic acid)-3,3-trimethyl-2-indolinylidenemethyl)cyclobutenediylium-1,3-diolate or any metal or quaternary nitrogen (ie. ammonium, mono-, di- or trialkylammonium, pyridinium etc) salt of the sulfonic acid.

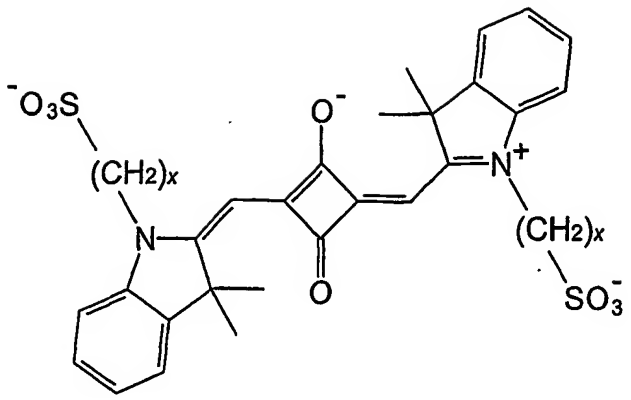
The present invention further provides a protein detector, which comprises a compound including a symmetrical skeletal structure of the general formula



in which *x* may represent any integer, and in which the phenyl rings may be substituted, in solution in water or a water containing solvent mixture, at concentrations from 1×10^{-9} to 1 moles per litre.

The present invention further provides a method for measuring the total dissolved protein content of a fluid sample, which method includes the steps of:

(a) dissolving a compound including a symmetrical skeletal structure of the general formula;



in which *x* may represent any integer, and in which the phenyl rings may be substituted, in solution in water or a water containing solvent mixture, at concentrations from 1×10^{-10} to 1 moles per litre.

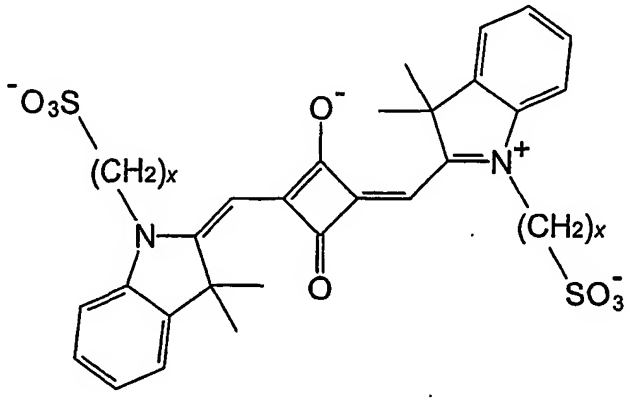
(b) admixing the solution of step (a) with a test fluid sample;

(c) measuring the fluorescence of the sample; and

(d) comparing the fluorescence with a standard value or values (ie. calibration plot), to obtain a value for the total protein content.

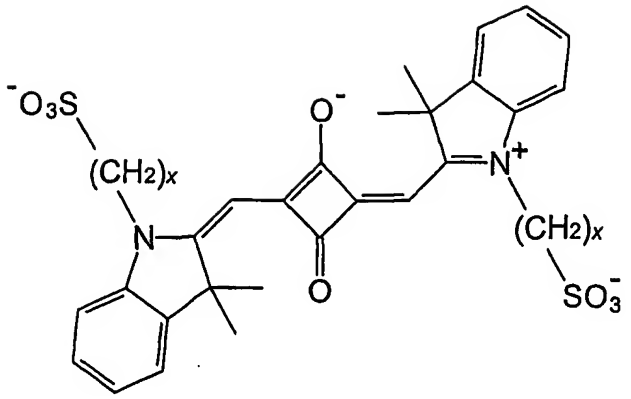
The present invention further provides a method for detecting and / or quantifying proteins separated electrophoretically in a supporting matrix, for example polyacrylamide, agrose or starch, either in the presence or absence of sodium dodecylsulfate (SDS), that has been fixed in an aqueous / organic / acid mixture comprising aqueous methanol and

acetic acid, wherein the matrix is subsequently stained with a photoluminescent compound including a symmetrical skeletal structure of the general formula;



in which x may represent any integer, and in which the phenyl rings may be substituted, in solution in 10% aqueous methanol or aqueous acetic acid, at a concentration of from 1 x 10^{-10} to 1 moles per litre, and destained to visualise bands.

In order to establish standard values for the fluorescence of aqueous solutions of proteins over the required detection ranges, the fluorescence of solutions containing known concentrations of the protein BSA, was measured using a 5 x 10^{-8} M aqueous solution of the compound including a symmetrical skeletal structure of formula:



where $x = 3$.

Dye 1

at an excitation wavelength of 624 nm ($\varepsilon = 1.0 \times 10^5$ $mol^{-1}cm^{-1}$) and a detection wavelength of 638 nm.

The results, for two separate calibration plots over the range 0-100 ng/mL BSA are shown in Figure 1; the results for three separate calibration plots, over the detection range of 0-500 ng/mL BSA are shown in Figure 2 and the results, for three separate calibration plots, over the detection range of 0-10 μg/mL BSA are shown in Figure 3.

It is particularly striking to note the linear response over these detection ranges.

It is also an advantage of the photo luminescent compounds of the present invention, that once a calibration curve has been constructed using BSA then any number of protein concentrations can be determined instantaneously. The fluorescence is also at a high

wavelength and is not masked by any diffuse lower wavelength organic fluorescence.

Examples

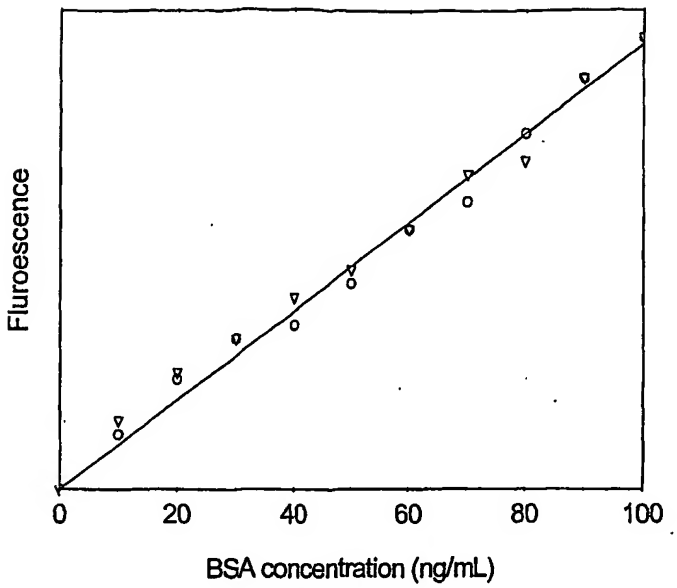


Figure 1

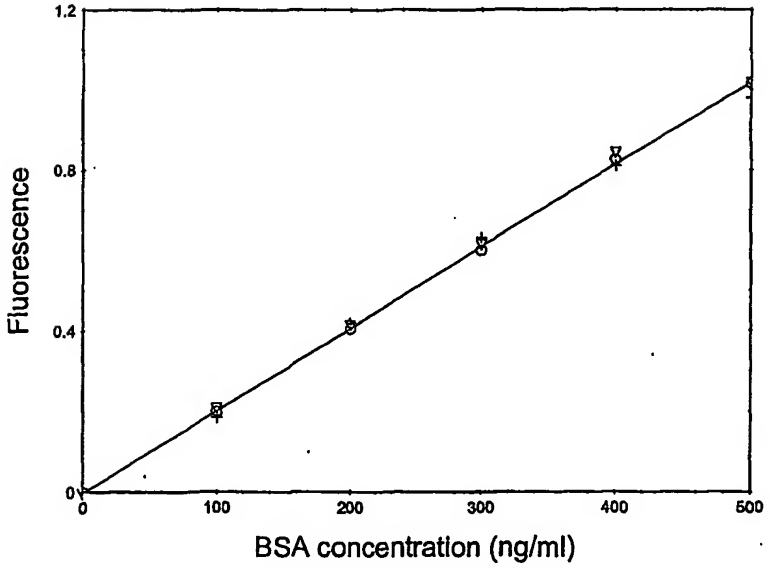


Figure 2

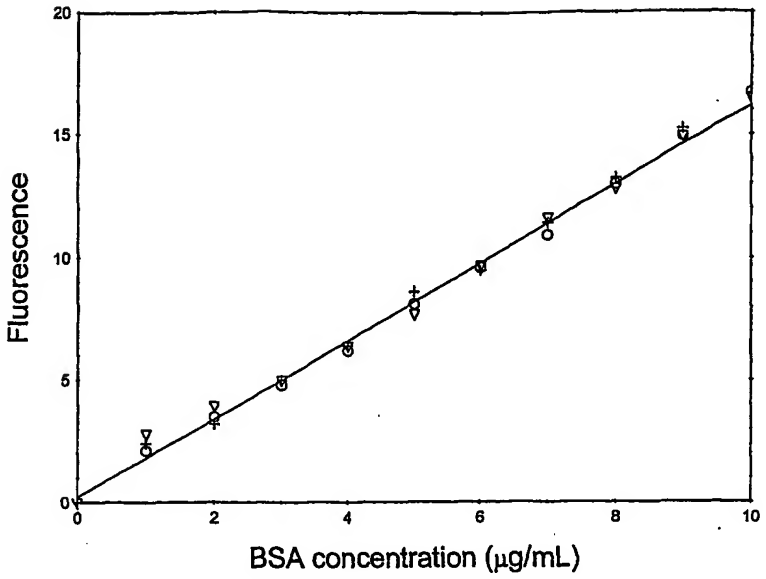
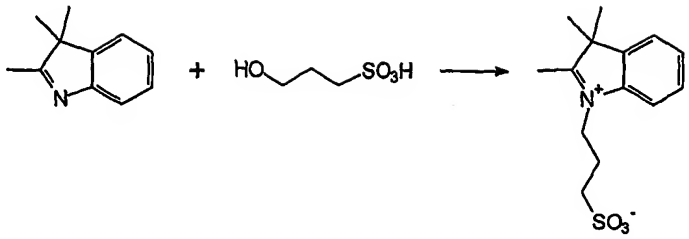


Figure 3

Experimental

Starting materials



2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine was prepared using the literature method of E. Havinga et al. in Synthetic Metals 69 (1995) pages 581-582 by heating 2,3,3-trimethylindolenine in excess 1,3-propanesultone and toluene, using a Dean and Stark apparatus. Upon cooling the excess solvent was decanted off and the product washed repeatedly with petroleum ether to yield a red oil. Crystals of 2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine formed from the oil upon standing for several weeks and the single crystal x-ray structure is shown in Figure 4.

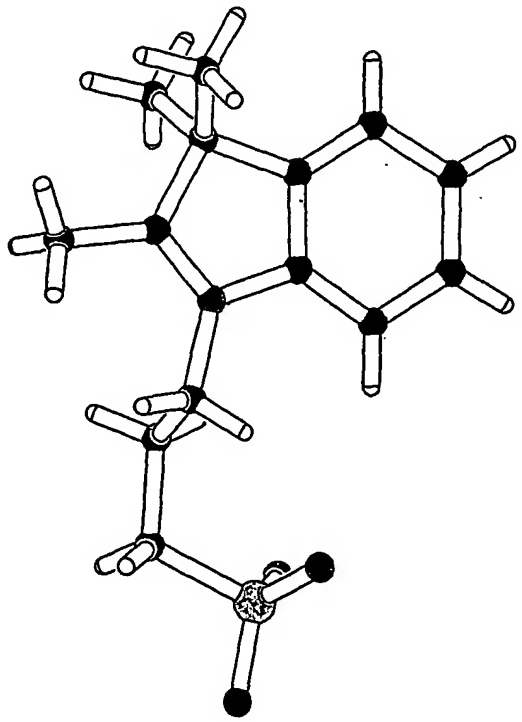
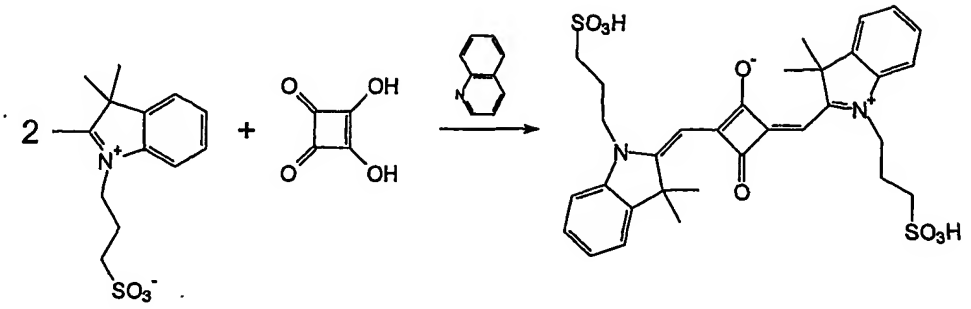


Figure 4

Molecular conformation and atom-naming scheme for the structure of 2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine.

Preparation of Dye 1



2,4-bis(1-(propan-3-sulfonic acid)-3,3-dimethyl-2-indolinylidenemethyl)cyclobutenebis(ylium)-1 3-diolate was prepared using the literature method of Sprenger and Ziegenbein in Agnew. Chem. Int. Ed. 6 (1967) page 533 by refluxing 2:1 molar amounts of 2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine and squaric acid with catalytic amounts of quinaline in 50/50 toluene/butan-1-ol using a Dean and Stark apparatus. The product was collected in vacuo after removal of the reaction solvents and repeated heated washings with petroleum ether.